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CORYNEBACTERIUM PYOGENES
A BIOCHEMICAL AND SEROLOGICAL STUDY

By
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HØI SØRENSEN, GUNNER: *Corynebacterium pyogenes. A biochemical and serological study.* Acta vet. scand. 1974, *15*, 544—554. — The study comprises 136 strains of Corynebacterium pyogenes originating from cattle (105), swine (20), sheep (1), and insects (10). For comparison 2 strains of human origin and 1 strain of Corynebacterium hemolyticum were examined.

One of the bovine strains was atypical, being gelatinase-negative, otherwise the strains of Cb. pyogenes were found to be biochemically identical apart from minor deviations in fermentation patterns (Table 1). Neither were antigenic differences demonstrated (gel diffusion analyses, Figs. 1 and 2).

Both of the human strains agreed biochemically with Cb. pyogenes (Table 1). By gel diffusion cross analyses one of them was found to be identical with Cb. pyogenes, the other not, though antigenically related to it (Fig. 2).

Cb. hemolyticum deviated biochemically as well as serologically from Cb. pyogenes, but the 2 organisms shared antigenic determinants (Fig. 3).

Corynebacterium pyogenes; fermentation reactions; antigenic characters; human infection; Corynebacterium hemolyticum.

Investigations on the biochemical characters of Cb. pyogenes are numerous, but the results are rather discrepant (see review by *Purdom et al.* 1958 and recent reports by *Aa & Müller* 1960, *Kielstein & Kötsche* 1963, *Jayne-Williams & Skerman* 1966, *Skovgaard* 1968, *Roberts* 1968, and *Narucka & Westendorph* 1972).

Reports on serological studies of Cb. pyogenes are relatively few (*Purdom et al., Hartwigk & Grund* 1960, *Matthews & Derbyshire* 1963, and *Kielstein & Kötsche* 1963, 1966), but agreeing in so far as no significant antigenic differences have been demonstrated.

Human infections with organisms similar to Cb. pyogenes have been described (*Purdom et al., Gärtner & Knothe* 1960, *Hartwigk* 1961, *Vega* 1970, *Zawahry et al.* 1972). However, the classification of these organisms was doubtful owing to lack of criteria for identification. By CAMP test *Záhorová & Kubelka* (1960) found that human strains, unlike animal strains, produced substances inhibiting the effect of staphylococcal α- and β-lysins on red blood cells, and this has been confirmed by several investigators (e.g. *Tauschnitz* 1964, *Kielstein & Kötsche* 1966, and *Narucka & Westendorph*). By immunodiffusion analyses the human strains studied by *Kielstein & Kötsche* were furthermore found to deviate antigenically from animal strains.

From the naso-pharynx of man, *Maclean et al.* (1946) isolated a corynebacterium resembling Cb. pyogenes, but not identical with it. The organism was named Corynebacterium hemolyticum, and like other human corynebacteria, it has been found to inhibit the action of staphylococcal lysins on r.b.c. (*Fraser* 1964, *Součková & Souček* 1972).

MATERIAL AND METHODS

The present study comprises 136 strains of Cb. pyogenes. Eighty-six strains originated in summer mastitis secretions, 19 in various other suppurations in cattle, 10 in insects (Hydrotaea irritans (Fall.), Simulium sp., and Culicoides sp., collected in summer mastitis areas, flying or on grazing cattle), and 20 in various suppurations in swine. For details about sources and isolation of these strains, see *Høi Sørensen* (1974). One strain (F 2) was from a lung abscess in a sheep.

For comparison 2 human strains (Strain 17835-68 = H 1 and Strain 25041-68 = H 2) classified as Cb. pyogenes and 1 strain of Cb. hemolyticum (ATCC 9345) were examined*. Strain H 1 originated in a wound necrosis and Strain H2 in an ear-cholesteatoma; both of them occurred in a mixed infection.

Media. Calf blood agar, serum gelatine, and nutrient broth: see *Høi Sørensen* 1973.

Basal medium for fermentation tests and W-S liquid culture medium (W-S medium): according to *Jayne-Williams & Skerman* 1966.

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* These strains were kindly supplied by Dr. W. Frederiksen, The State Serum Institute, Regional Department, Aalborg, Denmark.

Incubation. All cultures were incubated at 37°C; blood agar cultures in jars with approx. 10 % CO_2.

Storage of strains. For short term storage 48-hr. cultures on blood agar were kept in jars at 5°C. For prolonged storage 48-hr. blood agar cultures were suspended in skim milk and freeze-dried. Prior to examination the strains were subcultured in 3 passages on blood agar.

Catalase test: see *Høi Sørensen* 1973.

Fermentation reactions. To basal medium was added 1 % of the test substances and 4 % of a 0.2 % solution of phenol red.

Tubes containing 10 ml W-S medium were heated to 37°C, inoculated with 24-hr. blood agar culture, and incubated for 20 hrs. Tubes with 5-ml volumes of medium were heated to 37°C, inoculated with 2 drops of W-S medium culture, and incubated for 21 days. In every test a tube with basal medium and phenol red, but without test substance, was inoculated as control.

Readings were performed daily during the first week and subsequently at intervals of 2—3 days.

Liquefaction of serum gelatine. Tubes with 5 ml medium were inoculated with 24-hr. blood agar cultures and incubated for 48 hrs. Before reading the tubes were placed in the refrigerator for 1 hr.

CAMP test: see *Schalm et al.* 1971.

The 1 strain of Cb. hemolyticum, the 4 strains used for preparation of antisera, 10 bovine, 10 porcine, and the 1 ovine strain of Cb. pyogenes were examined.

Serological investigations. Antisera were prepared against Strains S 2 and S 50, both of which had been isolated from summer mastitis secretions, and against Strains H 1 and H 2.

Cells from 48-hr. cultures in W-S medium were washed 3 times in saline and suspended in saline with 0.2 % formalin (approx. 10^9 cells per ml according to the McFarland scale). Rabbits were given 9 intravenous injections at intervals of 2—3 days, doses increasing from 0.1 to 2.0 ml. After 2—8 months' resting periods the procedure was repeated, and 5 days after the last injection blood samples were collected by venous puncture.

Gel diffusion analyses. Lancefield extracts (*Lancefield* 1933) were prepared from 48-hr. cultures in W-S medium (Cb. pyogenes) or in nutrient broth (Cb. hemolyticum). The technique described by *Høi Sørensen* (1973) was used, except that the gel was Difco Special Agar-Noble (2 %) instead of Agarose Behringwerke (1 %). All strains were tested against anti-S 2 serum/S 2-antigen, and the strains examined by CAMP test were furthermore tested against anti-S 50, anti-H 1, and anti-H 2 serum and the respective homologous antigens.

RESULTS

Corynebacterium pyogenes

All strains formed hemolytic colonies on blood agar.

Microscopy showed Gram-positive or Gram-labile, small ple-

omorphic rods, in 24-hr. cultures in W-S medium frequently almost coccal, but in 24-hr. blood agar cultures typically rod-shaped.

All strains were catalase-negative, and all but 1 (R 8, originating from a summer mastitis secretion) liquefied serum gelatine.

Glucose, maltose, lactose, ribose, fructose, and xylose were fermented within 4 days by all strains (early fermentation). Also galactose and dextrin were early fermented by some strains, while others required 5—14 days (late fermentation). All strains showed late fermentation of saccharose, trehalose, and glycerol. As to mannose, inositol, and starch some strains showed late fermentation, while the color of the indicator in others was not

Table 1. Fermentation reactions of Corynebacterium pyogenes

	Origin of strains				
	cattle	swine	sheep	man	insects
Glucose	++	++	++	++	++
Maltose	++	++	++	++	++
Lactose	++	++	++	++	++
Ribose	++	++	++	++	++
Fructose	++	++	++	++	++
Xylose	++	++	++	++	++
Galactose	++/+(45)	++/+(8)	+	++	++/+(6)
Dextrin	++/+(49)	++/+(11)	+	++	++/+(7)
Saccharose	+	+	+	+	+
Trehalose	+	+	+	+	+
Glycerol	+	+	+	+	+
Mannose	+/w(16)	+/w(2)	w	+	+/w(2)
Inositol	+/w(23)	+/w(3)	+	w	+/w(3)
Starch	+/w(64)	+/w(6)	+	+	+/w(8)
Arabinose	w	w	w	w	w
Mannitol	—	—	—	—	—
Melibiose	—	—	—	—	—
Cellobiose	—	—	—	—	—
Raffinose	—	—	—	—	—
Salicin	—	—	—	—	—
Sorbitol	—	—	—	—	—
Number of strains	105	20	1	2	10

++ = early fermentation (acid produced after 1—4 days).
+ = late fermentation (acid produced after 5—11 days).
w = weak acid production, early or late.
Figures in brackets indicate numbers of strains showing late or weak acid production.

completely changed (weak acid production). Arabinose was weakly fermented by all strains, and none fermented mannitol, melibiose, cellobiose, raffinose, salicin or sorbitol. The results are listed in Table 1.

The 2 human strains and all the animal strains examined were found to be CAMP-negative or feebly CAMP-positive. None of them inhibited the action of staphylococcal β-lysin on calf r.b.c.

By cross analyses performed as gel diffussion tests the reference Strains S 2, S 50, and H 1 appeared to be identical, while Strain H 2 deviated from them, the precipitin lines showing re-

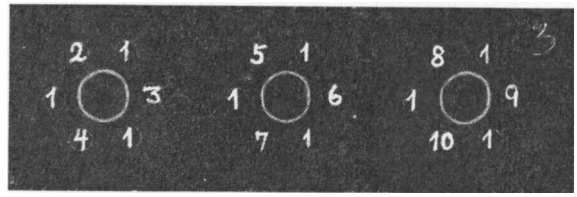

Figure 1. Gel diffusion reactions of anti-S 2 serum with extracts of Strain S 2 and 9 heterologous strains.
Center wells: Rabbit (No. 18) anti-S 2 serum.
Well 1: Extract of reference strains S 2.
Wells 2, 3, and 4: Extracts of 3 heterologous bovine strains.
Wells 5, 6, and 7: Extracts of 3 porcine strains.
Wells 8, 9, and 10: Extracts of 3 strains isolated from insects.

action of partial identity (Fig. 2). Between H 2-antigen and anti-H 1 serum only weak reaction of partial identity or no reaction at all was observed. When tested against anti-S 2, anti-S 50, anti-H 1, and anti-H 2 serum and the homologous antigens all strains conformed to Strains S 2 (Fig. 1), and all strains examined furthermore agreed with Strains S 50 and H 1, while none agreed with Strain H 2 (Fig. 2).

Corynebacterium hemolyticum

On blood agar the colonies showed some similarity to colonies of hemolytic streptococci. Microscopy of 24-hr. blood agar cultures showed Gram-positive, very pleomorphic and frequently rather coarse rods; in nutrient broth cultures they were even more pleomorphic.

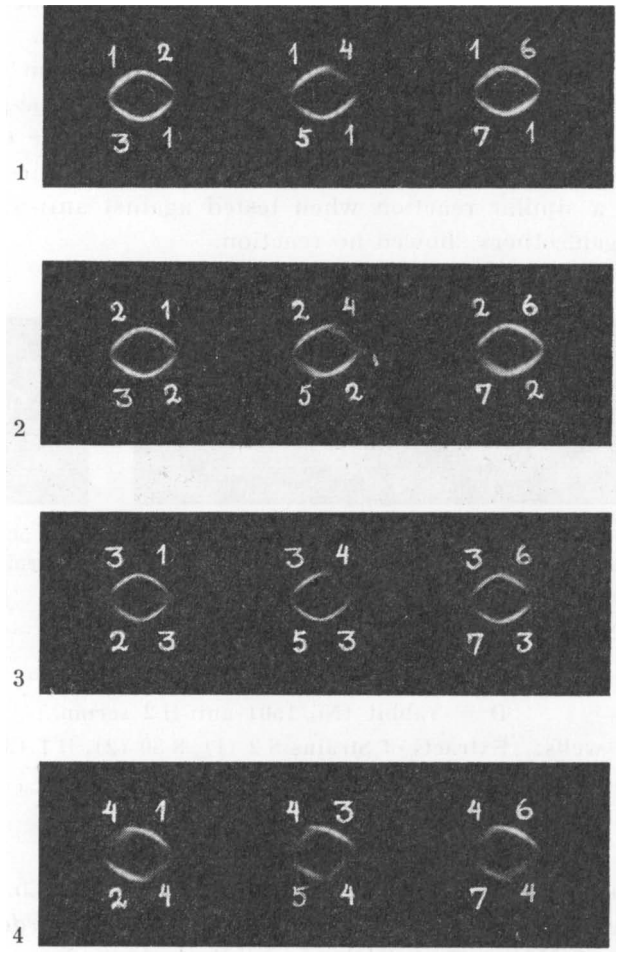

Figure 2. Gel diffusion reactions of anti-S 2, anti-S 50, anti-H 1, and anti-H 2 serum with extracts of the reference strains, 2 porcine strains (Gr. 37 and Gr. 46), and 1 ovine strain (F 2).

Center wells: Slide 1 = rabbit (No. 19) anti-S 2 serum.
Slide 2 = rabbit (No. 153) anti-S 50 serum.
Slide 3 = rabbit (No. 149) anti-H 1 serum.
Slide 4 = rabbit (No. 150) anti-H 2 serum.

Outer wells: Extracts of Strains S 2 (1), S 50 (2), H 1 (3), H 2 (4), F 2 (5), Gr. 37 (6), and Gr. 46 (7).

In CAMP tests the development of staphylococcal β-lysin zones was inhibited. Serum gelatine was liquefied after prolonged incubation (3—5 days), while catalase was not produced.

Fermentation reactions could not be studied by the methods employed, as growth was scanty in the W-S medium.

By gel diffusion tests extracts of Cb. hemolyticum were precipitated by anti-S 50, anti-H 1, and anti-H 2 serum, the precipitin lines forming patterns of partial identity with those of the respective homologous strains (Fig. 3). Some antigen preparations showed a similar reaction when tested against anti-S 2 serum/ S 2-antigen, others showed no reaction.

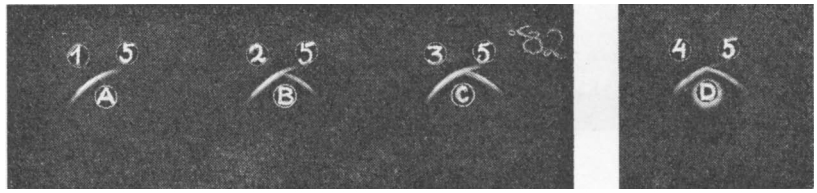

Figure 3. Gel diffusion reactions of anti-S 2, anti-S 50, anti-H 1, and anti-H 2 serum with extracts of the 4 reference strains and of Corynebacterium hemolyticum.

Antibody wells: A = rabbit (No. 19) anti-S 2 serum.
B = rabbit (No. 153) anti-S 50 serum.
C = rabbit (No. 149) anti-H 1 serum.
D = rabbit (No. 150) anti-H 2 serum.

Antigen wells: Extracts of Strains S 2 (1), S 50 (2), H 1 (3), H 2 (4), and Cb. hemolyticum (5).

DISCUSSION

In previous studies it was found that strains of Cb. pyogenes differ considerably in fermentation reactions (*Purdom et al.* 1958, *Aa & Müller* 1960, *Kielstein & Kötsche* 1963, *Jayne-Williams & Skerman* 1966, *Skovgaard* 1968, *Roberts* 1968, and *Narucka & Westendorph* 1972). *Aa & Müller* reported that even the same strain was inconstant in its biochemical characters in repeated tests. This conflicts with the results of the present study, in which the fermentation features of Cb. pyogenes appeared to be constant, except that the time required for some reactions varied (galactose, dextrin), and that in other reactions (mannose, inositol, starch) the color of the indicator was not completely changed by all strains (Table 1). The discrepant results of previous studies may be ascribed to the use of different methods of investigation. In the present study the media and technique described by *Jayne-Williams & Skerman* were used, with the

modifications that cultures in W-S medium were used for inoculation of the test tubes instead of washed organisms grown in carbohydrate-free media, that the indicator was phenol red instead of bromothymol blue, and that the tests were observed for 3 weeks instead of 24 hrs. The W-S medium was found to be very suitable for culturing Cb. pyogenes, and the method employed ensured approximately uniform inocula with respect to growth phase and density of organisms. With the W-S medium, which contained 1 % glucose, minimal amounts of that carbohydrate were added to all the test tubes, and this may have influenced the results. However, the color of the indicator never changed in the control tubes containing no test substrate. The wash of organisms prior to inoculation was omitted, as Cb. pyogenes seems to be rather labile.

The gelatinase-negative Strain R 8 conformed in all other respects to the rest of the strains of Cb. pyogenes, and seemed to agree with the atypical strains described by *Hartwigk & Grund* (1960) and by *Kielstein & Kötsche* (1966).

In agreement with the results of *Kielstein & Kötsche* (1963, 1966), gel diffusion analyses were found to be valuable for the serological identification of Cb. pyogenes. Animal strains appeared to be antigenically homogenous, which agrees with the results of *Hartwigk & Grund, Matthews & Derbyshire* (1963), and *Kielstein & Kötsche* (1963, 1966).

The 10 strains isolated from insects fulfilled the biochemical as well as the serological criteria for identification of Cb. pyogenes. The insects in question had been collected in summer mastitis areas, and from some of them also Micrococcus indolicus was isolated (*Høi Sørensen* 1973, 1974).

Both of the human strains (H 1 and H 2) agreed biochemically with the animal strains, and none of them inhibited the action of staphylococcal β-lysin in CAMP tests. By gel diffusion cross analyses the identity of Strain H 1 with Strains S 2 and S 50 was verified (Fig. 2). This would seem to be the first time a human strain has been definitely identified as Cb. pyogenes. Strain H 2 was found to be antigenically related to, but not identical with Strains S 2 and S 50, while the reaction of H 2-antigen with anti-H 1 serum was inconstant (Fig. 2). Against anti-H 2 serum/H 2-antigen extracts of strains of Cb. pyogenes showed a reverse reaction of partial identity, indicating that anti-H 2 serum contained a broader spectrum of Cb. pyogenes precipitins than re-

vealed by H 2-antigen. This type of reaction has not been observed in experiments with other polysaccharide antigen/antibody systems. Possibly Strain H 2 is a human-adapted animal strain.

While it was confirmed that (as described by *Fraser* 1964 and by *Součková & Souček* 1972) Cb. hemolyticum would inhibit the action of staphylococcal β-lysin on r.b.c., Cb. pyogenes was found not to have this effect, and the 2 organisms were therefore easily distinguished by CAMP test. Furthermore Cb. hemolyticum was found to differ biochemically as well as antigenically from Cb. pyogenes. Yet, the 2 organisms share antigenic determinants (Fig. 3).

CONCLUSION

Cb. pyogenes possesses a number of constant fermentation characters and can be identified serologically by gel diffusion analysis. Strains from different animal sources seem to agree biochemically as well as antigenically.

Cb. pyogenes and an organism antigenically related to it may occur in pathological conditions in man.

Cb. hemolyticum shares antigenic determinants with Cb. pyogenes.

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SAMMENDRAG

Corynebacterium pyogenes.
En biokemisk og serologisk undersøgelse.

Undersøgelsen omfatter 136 stammer af Cb. pyogenes isoleret fra kvæg (105), svin (20), får (1) og insekter (10). Til sammenligning blev undersøgt 2 stammer af human oprindelse samt én stamme af Corynebacterium hæmolyticum.

Én af de bovine stammer var atypisk, idet den ikke smeltede serum-gelatine. Bortset fra mindre afvigelser i forgæringsmønster (tabel 1) fandtes Cb. pyogenes stammerne i øvrigt identiske med hensyn til biokemiske og serologiske egenskaber (gel diffusions analyse, figur 1 og 2).

Begge humane stammer var i biokemisk henseende i overensstemmelse med Cb. pyogenes (tabel 1). Ved gel-diffusions kryds-analyse fandtes den ene identisk med Cb. pyogenes, den anden ikke identisk, men antigent beslægtet med den (figur 2).

Cb. hæmolyticum afveg såvel biokemisk som serologisk fra Cb. pyogenes, men havde antigene determinanter fælles med den (figur 3).

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